7

Chapter 7

General discussion

Bacteria are typically seen as individual planktonic cells, dividing by binary fission to achieve rapid growth. However, many bacteria prefer to live in a community, either during a defined stage of their life cycle in the form of a biofilm, or permanently as a full-fledged multicellular organism (Claessen *et al.*, 2014). Living as a community increases fitness by offering better protection and allowing more complex behavior like division of labor (Jefferson, 2004). But in order for neighboring cells to work in unison also more complex structures are required, like adhesion and cell-cell communication.

Streptomycetes are mycelial organisms that grow as hyphae, and they undergo a complex life cycle whereby they propagate via sporulation, involving complex regulatory networks and developmental checkpoints (Flärdh & Buttner, 2009). Different parts of the mycelia take up different roles. The tip of the hyphae grow by extension, and at these apices a wide range of cellulolytic and proteolytic enzymes is secreted, allowing assimilation of dead plant matter (Willemse *et al.*, 2012). More centrally positioned sections produce a wide variety of antibiotics, for which the species is best recognized (Bibb, 1996). In view of all the complex mechanisms being directed throughout the mycelia we can truly appreciate the multicellularity of this organism. Having said that, from the perspective of commercialization this complex morphogenesis also poses several major issues, which was the driving force behind this thesis.

Streptomyces and Biotechnology

From an applied perspective we are very interested in *Streptomyces* species *because* they produce such a wide chemical variety of compounds that we apply as antibiotics, antifungal,

anticancer and immunosuppressant agents (Hopwood, 2007). Also the large diversity of enzymes are put to good use, finding uses in the washing industry to the pretreatment of lignocellulosic materials for bioethanol production.

Industrial production of these compounds generally occurs in large bioreactors where the micro-organisms are cultivated in a fluid environment. The liquid is mixed vigorously to allow aeration and to secure an even distribution of biomass and nutrients. Although this is an efficient use of space and equipment it is very different from the natural soil habitats. Especially multicellular organisms like filamentous fungi or *Streptomyces* are a poor match for submerged fermentation (Wucherpfennig *et al.*, 2010). The mycelial network entangles in the turbulent environment changing the liquid in a non-Newtonian fluid, which increases the apparent viscosity, negatively affecting the mass transfer and mixing times, thereby decreasing the efficiency of the fermentation (Metz *et al.*, 1979). Also the long hyphae themselves are prone to breaking, causing lysis of compartments (Li *et al.*, 2002). Besides growing as a loose mycelial network, many *Streptomyces* species can aggregate into dense pellets. Pellets do not affect the apparent viscosity nearly as much, but also limit the maximal obtainable rates by restricting mass transfer toward the pellet's core. Interestingly pellets also play a regulatory role in production, sometimes impacting product formation in a yet poorly understood mechanism (Wardell *et al.*, 2002, López *et al.*, 2005). Clearly, the efficiency of a fermentation with a filamentous micro-organism depends greatly on its morphology. Work described in this thesis aims at the discovery, understanding and development of novel ways to control morphology of *Streptomyces* species with the goal of improving the fermentability and production. An overview of what is known about the environmental and genetic factors affecting liquid morphogenesis is discussed in Chapter 2.

Reverse engineering as a source of new morphogenes

Our aim to improve the fermentability of *Streptomyces* prompted analysis of *S. lividans* strains PM01 and PM02 that had been selected for their favorable growth behavior in a bioreactor, obtained through evolution in a chemostat (Roth *et al.*, 1985). Over the course of the evolution experiment the strains adapted to the environment, resulting in the stable strains PM01, which makes very small pellets, and PM02, which is unable to make any pellets at all. Both had superior maximum growth rates compared to the parental strain, but were genetically black boxes. As these strains descended from *S. lividans* 66*,* which is a preferred heterologous enzyme production host because of a low proteolytic activity and the ability of secreting complex enzymes (Anné *et al.*, 2012), we initially considered PM02 as a potential production platform only to find that it was unable to secrete enzymes through the twin arginine transporter (Tat) system. This made this strain a very attractive candidate to reverse engineer. With reverse engineering we aimed to reconstruct the key features of these black box mutants by targeted genomic disruption. Sequencing of PM01 and PM02 revealed a handful of mutations that were sub sequentially recreated in the parent strain leading to the discovery of *matA* and *matB*, two genes that were essential for

mycelial aggregation. We compared the growth of clean knockout mutants and found that the absence of pellets in the *matAB* double mutant increased the growth rate and enzyme production rate by 60%, both most likely the result of an increased surface area exposed to the media, allowing high substrate uptake rates. A major advancement was achieved with this discovery, improving *S. lividans* as a production host for enzymes, but also paving the way to an increased understanding and control of morphogenesis in liquid-grown cultures.

Mechanism of pellet aggregation

The discovery of the mat locus was not our first adventure into genetic morphology engineering. Overexpression of the developmental protein SsgA induces septum formation throughout the vegetative mycelium, which increases the rate at which fragmentation takes place, leading to a reduced particle size (van Wezel et al., 2000a, van Wezel et al., 2006). Similar to the mat mutants, reduction of particle size resulted in increased growth rate of the culture and increased enzyme production rates. Also the cslA-glxA gene cluster (Xu et al., 2008, Chaplin et al., 2015) and the recently discovered partner dtpA (Petrus et al., 2016)2016 are somehow involved in controlling Streptomyces morphology in liquid-grown cultures. These genes are responsible for the production of a cellulose-like polysaccharide that, together with the hydrophobic Chaplin proteins, facilitate adhesion (de Jong et al., 2009b). Beside this there are reports that extracellular DNA (Kim & Kim, 2004), cell wall fusions (Koebsch et al., 2009) and hyaluronic acids (Kim & Kim, 2004) are involved in this process of cellular adhesion. Similar to the surface attachment of a biofilm the Streptomyces liquid morphogenesis is a complex process.

In Chapter 4 we showed that the MatB protein produces the exo-polysaccharide poly-1,6-β-N-acetylglucosamine (PNAG), which is well characterized and an essential component of cellular adhesion in E. coli, S. epidermidis and B. subtilis (Wang et al., 2004, McKenney et al., 1998, Roux et al., 2015). The polysaccharide, synthesized by the glycosyltransferase domain of MatB, acts as a bacterial glue; the extracellular carbohydrate esterase domain of MatB most likely partially deacetylated the chain, giving it a positive charge allowing it to stick to the outside of the cell wall. This layer can be seen by high resolution scanning electron microscopy covering the entire cell wall. Interestingly, great similarities exist between the morphology in liquid cultures of null mutants deleted for either the matAB or cslA-glxA genes. We found that MatA and MatB are required to support adhesion to hydrophilic surfaces like glass (and cells), while CslA is required for adhering to hydrophobic surfaces like polystyrene. This opens the idea that the architecture of a pellet depends on both hydrophilic and hydrophobic adhesive forces. Interestingly, in liquid-grown cultures the expression of the mat genes to non-pelleting strains such as S. venezuelae, S. clavuligerus, S. albus or Sacch. erythraea was able to induce pellet formation, which suggest that under non-native conditions the presence of PNAG is enough to induce pellet formation. For now it remains unclear why native aggregation depends on a combination of mechanisms, but possibly it is a matter of robustness.

Gateway to synthetic morphology

Understanding the mechanism by which the *mat* genes act led to the insight that it has a very direct way of facilitating adhesion. This led to the idea that these genes could be employed rationally to improve the fermentability of *Streptomyces*. In Chapter 5 strains were created that expressed *matA* and *matB* from different promoters with the goal of allowing initial fast dispersed growth combined with later aggregation during exponential growth. The promoter regions of *chpE* (Claessen *et al.*, 2003) and *glpQ2* (Thomas *et al.*, 2012) were selected for their expression profile based on previous transcription data (Nieselt *et al.*, 2010) and fusion with the *matAB* locus gave strains that had a growth rate that was on par with the *matAB* null mutant described in Chapter 3, but which limited the rise of apparent viscosity also associated with full dispersed growth. Interestingly, although the viscosity as a function of morphology had been characterized for the filamentous fungus *Absidia corymbifera* (Kim *et al.*, 1983), this had never been done for *Streptomyces*. We found that, compared to the data from filamentous fungi, the dispersed growing *S. lividans matAB* null mutant affected the flow behavior already at biomass concentrations as low as 1 g/L, creating a non-Newtonian fluid, but the rise in apparent viscosity was not as quick during the fermentation process. Most likely this is the result of fragmentation as image analysis showed that particles of the *matAB* null mutant remain small. Pellets on the other hand affect viscosity only at biomass concentrations not readily encountered in fermentations. By analyzing multiple particles through image analysis we could establish that strains with altered *matAB* expression obtain morphologies that are best described as the intermediate between the two extreme morphologies of pelleting and dispersed growth. Over time the strains progress towards a pelleting morphology, but not really aggregating to the same extent as wild-type *S. lividans*. It is an interesting notion that potentially large amounts of PNAG are needed to allow wild-type levels of aggregation. Still we believe that the example of morphology engineering that we describe in this chapter is a major step forward in the fermentability of streptomycetes.

Streptomyces in microcultures

Besides being difficult to control in shake flasks or bioreactors, growth of *Streptomyces* on a small scale is probably even more difficult (Sohoni *et al.*, 2012). The heterogeneity of a population consisting of multicellular aggregates (van Veluw *et al.*, 2012), combined with a complicated interconnectedness of morphology and environment creates difficulties for small scale growth, while growth on such a scale is desirable for high throughput experiments needed for screening thousands of strains under many environmental conditions needed to explore the chemical diversity offered by these strains. As discussed in Chapter 2 morphology is the result of genetic and environmental factors. In Chapter 6 we show that it is possible to modulate the morphology of *Streptomyces lividans* and *Streptomyces coelicolor,* both of which are extreme pellet formers, by cultivating them in microtiter plates on a vortex. This

allowed precise speed control at extreme agitation speeds, matching shear forces found in larger cultivation vessels. Interestingly, at low agitations rates (around 800 rpm) aggregation was absent, and pellet formation could be induced only by increasing the mixing rate to values of at least 1000 rpm. A substantial mixing rate is needed to induce the fragmentation rate, for which purpose coiled springs are routinely added to shake flasks. Increasing the stirring speed further to 1600 rpm fragmentation became more dominant, with abundant lysis seen in the form of small debris. Our data show that the mycelial morphology was optimal for these growth conditions at 1400 rpm mixing rate, which gave morphological characteristics close to those found in shake flasks. It provides important proof of concept that it is feasible to screen for enzyme and antibiotic production in volumes as small as 100 µL, which is an important step forward in the screening of streptomycetes.

OUTLOOK: LINKING MORPHOLOGY AND PRODUCTIVITY

As a result of interdependencies between morphology, environment and genetics, and the interconnection of morphology and productivity, an interdisciplinary out of the box approach is needed to fully elucidate all the actors and understand how they affect production. Therefore this thesis does not only describes multiple perspectives in understanding and controlling *Streptomyces* morphology in liquid cultures, but it can also be set in a larger framework where we aim to understand the morphogenesis on multiple levels, ranging from studying in detail the role of cell division (Celler *et al.*, 2016), following the dynamics of cellular processes in a time-resolved manner (Willemse *et al.*, 2012) and modeling hyphal growth in silico (Celler *et al.*, 2012). The next challenge is to converge the lessons learned from the different approaches and relate it to further optimization of the production process.

From experiments performed on solid media it is known that antibiotic production and programmed cell death in the vegetative mycelium are part of the developmental cycle, hypothetically to create nutrients and a competitor-free environment for the formation of spores (Manteca *et al.*, 2005, Wildermuth, 1970, Miguélez *et al.*, 1999). The core of a pellet has a characteristic; some antibiotics are produced there and cell death is also present (Manteca *et al.*, 2008). This, combined with a limited amount of studies in liquid cultures led to the idea that pellets are beneficial for antibiotic production (van Wezel *et al.*, 2006, Martin & Bushell, 1996, López *et al.*, 2005). As a result of the poor solubility of oxygen in water, it is most likely that this is the cue that orchestrates this. In biofilm systems the absence of oxygen is a trigger for developmental genes (Worlitzsch *et al.*, 2002). Also oxygen-limiting conditions have actually been measured for *Aspergillus niger* in pellets as small as 200 µm (Hille *et al.*, 2005), which makes it likely that *Streptomyces* pellets also possess an anoxic core. Although the link between pellet formation, oxygen limitation and development requires further elucidation there is plenty of evidence that oxygen limitation is a major player in this.

We have performed some initial experiments that showed the effects of oxygen limitation

on the activity of the *mat* genes. The non-aggregating mycelium seen under low mixing conditions in the micro cultures described in Chapter 6 are likely the result of low oxygen conditions as similar morphologies are obtainable by restricting the air flow in a shake flask. Additions of a nitric oxide donor, an important cue for anaerobic metabolism (Barraud *et al.*, 2006, Plate & Marletta, 2012), also inhibited pellet formation and repression of the *mat* promoter. This is again similar to known biofilm systems where it is also known that exo-polysaccharide production is inhibited by nitric oxide (Barraud *et al.*, 2009). Interestingly, this regulation is performed though c-di-GMP in other organisms by direct interaction of the glycosyltransferases. Likely in streptomycetes it occurs through a different mechanism as bioinformatics analysis suggest that c-di-GMP binding PilZ domain is absent throughout the genus. In *Streptomyces* c-di-GMP acts as a signaling molecule in development, by modulating the activity of the global developmental regulator BldD (Tschowri *et al.*, 2014). It will be interesting to see if c-di-GMP also affects exo-polysaccharide production.

Work described in this thesis has offered new ways to tune the mycelial morphology, which enable us to resolve the link between morphology and production in the near future, eventual leading towards an optimal morphology for productivity. We are now able to regulate pellet size through *matAB* gene activity, creating populations with altered pellet sizes. Also as discussed in Chapter 4, introduction of the *mat* genes induces pellet formation in non-pelleting species and, although the research is still in progress, initial trials with these strains did result in altered growth profile, associated with pelleted growth, but also a delayed production of antibiotic production. This could mean that in liquid-grown cultures the occurrence of antibiotic production is not necessarily linked to the morphology and that there is still a lot we do not understand in terms of how antibiotic production is regulated.

The work presented in this thesis has provided new insights into the genetic and environmental factors that control the morphology of Streptomyces in submerged cultures, and it also offers leads as to how this can be harnessed to fine tune the growth parameters for improved productivity. With that, new paths have been uncovered that may lead towards better understanding of the principles of mycelial growth of streptomycetes, as well as new opportunities for their use as commercial production platform.


7